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Breast Cancer Diagnosis Using N_2 Laser Excited Autofluorescence Spectroscopy

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Background and Objective: **This article reports results of an in vitro study involving 63 patients for the evaluation of the diagnostic potential of N_2 laser excited autofluorescence spectroscopy of human breast tissues.**
Materials and Methods: **The N_2 laser-excited spectra were recorded from benign (fibroadenomas, 35 patients), cancerous (ductal carcinomas, 28 patients), and normal (the uninvolved areas of the resected cancerous specimens). A stepwise multivariate linear regression (MVLR) analysis was developed to analyze the diagnostic content of the breast tissue fluorescence spectra.**
Results: **Significant changes were observed in the autofluorescence from normal, benign, and cancerous breast tissues, particularly in the spectrally integrated fluorescence intensity. The ratio of mean spectrally integrated intensity from cancerous tissues to that from benign tumor and normal tissues were 3.2 and 2.8, respectively. A discrimination parameter based on spectrally integrated intensity alone provided a sensitivity and specificity of up to 99.6% over the sample size investigated for discrimination of cancerous breast tissues from benign/normal.**
Conclusion: **Our results suggest that a straightforward measurement of the total integrated fluorescence intensity can provide excellent discrimination between cancerous and benign/normal breast tissues. Lasers Surg. Med. 21:417–422, 1997.**

Key words: breast cancer; autofluorescence spectroscopy; ductal carcinoma; fibroadenoma; MVLR analysis

INTRODUCTION

Cancer of breast is one of the most common malignant tumors among women. Good screening methods for breast cancer are therefore of considerable interest. X-ray mammography, the best available means of detecting breast cancer at present has two important drawbacks. First, it leads to a very large number of false positives, i.e., a very large proportion (60–90%) of mammographically abnormal detections turn out to be benign upon invasive breast biopsy [1], leading to avoidable trauma and psychological stress to patients. Secondly, frequent exposure to ionizing x-ray radiation during mammography has potential hazards, howsoever remote. Therefore, there exists considerable interest in the development of alternate methods for diagnosis based on optical spectroscopic techniques [2].

Alfano and his co-workers were the first to show that significant differences exist in the static [3] and time-resolved fluorescence [4] of malignant and normal human breast tissues. While their earlier work used visible argon-ion laser lines, and the second and third harmonic of Nd-glass laser [3,5] and exploited visual spectral differences for diagnostics, the latter work [6,7] used 300 nm excitation and the ratio of fluorescence intensities at 340 nm and 440 nm as the diagnos-

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tic index, and could discriminate malignant from benign and normal breast tissues. It is pertinent to note that a compact and less expensive N_2 laser is more convenient to use in a clinical situation, and although it has already been used for investigations on colonic and cervical tissues [8,9], to our knowledge, no report exists on its use for studies on human breast tissues. We have therefore investigated N_2 laser excited autofluorescence spectra from cancerous and adjoining normal human breast tissues, along with other tissues from uterus [10] and colon. The N_2 laser excited autofluorescence spectra from the breast tissues showed, as expected, significant differences from those obtained with 300 nm or other longer wavelength laser excitation. Further, the spectra of breast tissues were found to differ considerably from that of colon and uterine tissues.

In this article, we present the results of our in vitro studies on N_2 laser excited autofluorescence from pathologically characterized human breast tissues obtained after resection from 63 patients, 28 with ductal carcinomas and 35 with fibroadenomas. The normal tissue samples were the uninvolved areas of the resected carcinoma samples. Significant differences in the spectral intensity distribution were observed for normal, benign, and cancerous tissues. In particular, a large difference was observed in the fluorescence intensity of cancerous tissues, adjoining normal tissue, and benign tumor tissues with the ratio of the spectrally integrated fluorescence intensity from cancerous site to that from the benign tumor and normal tissue site being ~3.2 and ~2.82, respectively. It is pertinent to note that although differences in the fluorescence intensity of cancerous tumor, benign tumor, and normal tissues have earlier been observed by Alfano and co-workers using different excitation wavelengths [3], these were not exploited for discrimination. Our analysis showed that a discrimination parameter, based on the value of the spectrally integrated fluorescence intensity alone, provided sensitivity and specificity value for cancer of ~99.6% over the sample size investigated. Our results therefore suggest that a straightforward measurement of the total integrated fluorescence intensity, as opposed to the analysis of the fluorescence spectra, can provide excellent discrimination.

MATERIALS AND METHODS

Pathologically characterized full thickness tissue samples were obtained from M.Y. Hospital, Indore, India and Choithram Hospital and Research Centre, Indore, India immediately after resection at surgery from 63 patients with breast tumor. The tissue samples were brought and stored in ice until study. The benign breast tissues were fibroadenomas (35 patients) and the cancerous tissues were ductal carcinomas (28 patients) with involvement of lymphatic channels and adjacent skin. The normal tissue samples were from the uninvolved areas of the resected cancerous tissues. These were yellowish in appearance and had profuse fat. Both the cancerous and the benign tissue samples could be grouped into two categories on gross visual examination. One group of tissue samples (Type-A) were firm and whitish in appearance, while the other group (Type-B) comprised of tissue samples which were soft and grayish.

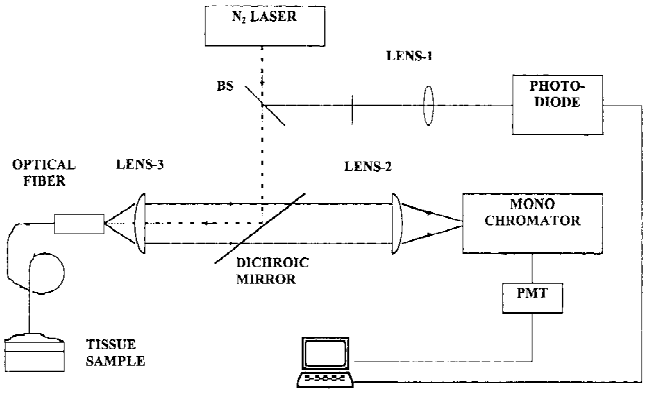

Fig. 1. A schematic diagram of the experimental set-up for N_2 laser excited autofluorescence spectroscopy of tissues.

Laser induced fluorescence spectra of the tissue samples were recorded using the experimental set-up shown in Figure 1. It uses a home-built pulsed N_2 laser emitting 7 nsec pulses with a repetition rate of 10 Hz and pulse energy of 200 μJ which is coupled to an optical fiber (core diameter 400 μm) via a dichroic mirror which reflects N_2 laser radiation (337 nm) and transmits the longer wavelength fluorescence output. The power of the laser pulse is monitored by a beamsplitter-photodiode combination. The fluorescence from the tissue, kept in contact with the fiber, is collected by the same fiber and imaged on the entrance slit of a scanning monochromator. The wavelength dispersed light at the exit slit of the monochromator is detected by a photomultiplier tube detector. A microprocessor based system developed in-house was used for on-line acquisition of N_2 laser power and fluorescence spectral data. The fluorescence spectra were measured from a

total of 911 sites, of which 436 sites were from benign tumor samples, 245 sites from cancerous tissue samples, and 230 sites from adjoining normal tissue. The fluorescence intensity from the same tissue site was observed to decrease with time over the first 3–4 hours of tissue removal and then stabilize. No significant differences were observed between the different tissue types in this respect. The measurements reported here were performed within 4–24 hours of tissue removal. A single spectrum was obtained from each site. Each site has been treated separately and classified on the basis of its spectra. All spectra were system response corrected. Before each measurement on tissue fluorescence, a spectrum was also acquired with the fiber placed on the face of a quartz cuvette containing a solution of known concentration of Rh-6G in ethyl alcohol. This served as the reference standard for calibration of intensity measurements.

In order to compare with the previous reports from Alfano's group, the 300 nm and the 488 nm excited autofluorescence spectra were also recorded using a commercial spectrofluorometer (SPEX, Fluorolog 2, Edison, NJ, U.S.A.). The excitation light, provided by a 450 W xenon lamp, was incident perpendicular to the tissue surface, and the light emitted was collected at approximately a 20° angle with respect to the excitation light.

RESULTS AND DISCUSSION

The instrument response corrected mean spectra of breast tissue samples excited with N_2 laser are shown in Figure 2. All the N_2 laser excited emission spectra (from benign, cancerous, and adjoining normal breast tissues) were characterized, in general, by two major wavelength bands centered at around 390 nm and 430 nm, respectively, and a shoulder around 520 nm which is most pronounced in cancerous breast tissues spectra. The fluorophores responsible for the 390 nm, 430 nm, and 520 nm bands are believed to be the structural proteins (collagen and elastin), the co-enzyme NADH/NADPH, and flavins, respectively. This was supported by the fact that the excitation spectra corresponding to these emission bands resemble the absorption spectra of the corresponding fluorophores. Our studies on excitation spectra and time-resolved measurements on 390 nm, 430 nm, and 520 nm bands suggest that the enhanced fluorescence yield for cancerous tissues is primarily due to enhanced concentration of the fluorophores. Details of these

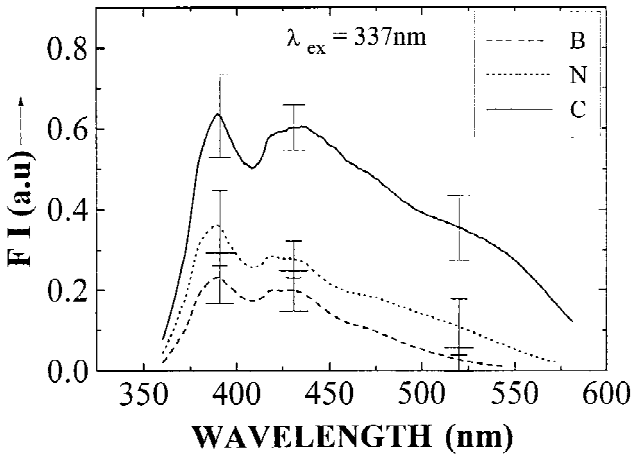

Fig. 2. Mean spectra from 245 cancerous (C), 230 normal (N), and 436 benign (B) breast tissue sites excited with N_2 laser.

studies will be published elsewhere. As is apparent from Figure 2, the spectrally integrated fluorescence intensity was observed to be much higher from cancerous sites compared to benign tumor and normal breast tissue sites. In Figure 3, we show a histogram for the spectrally integrated intensity for paired cancerous and adjoining normal samples from each of the 28 patients. The values shown represent the average over the sites (6–10) investigated in the given tissue sample. In Figure 4, we show the scatter plot of the integrated fluorescence intensity for paired cancerous and adjoining normal tissue samples from the first 10 patients included in the histogram shown in Figure 3. The considerable difference in the values of cancerous and normal tissue spectra is apparent. No significant difference was observed in the values for the ratio of standard deviation to the mean values for the two tissue types. The ratio of integrated fluorescence intensity of cancerous to adjoining normal breast tissues (I_C/I_N) was ~2.82, and that for cancerous to benign tumor tissues (I_C/I_B) was ~3.23. Even with the 488 nm excitation, the spectrally integrated fluorescence intensity was considerably higher for cancerous, as compared to benign tumor or normal tissues, with the ratio being I_C/I_N ~2.41 and I_C/I_B ~ 2.77. However, in contrast to reports from Alfano's group [3], no Raman peaks and subsidiary maxima were observed in our spectra, and the spectra were characterized by a single broad spectral band peaking at around 550 nm with a shoulder at around 575 nm.

For 300 nm excitation, the ratio of fluorescence intensities at 340 nm and 440 nm has been used by Alfano's group for discriminating cancer-

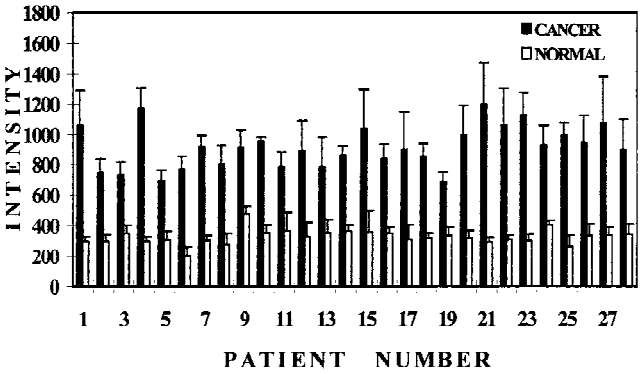

Fig. 3. Histogram for the spectrally integrated intensity for paired cancerous and adjoining normal breast tissue samples from each of the 28 patients. The values shown represent the average over the sites investigated in the given tissue sample.

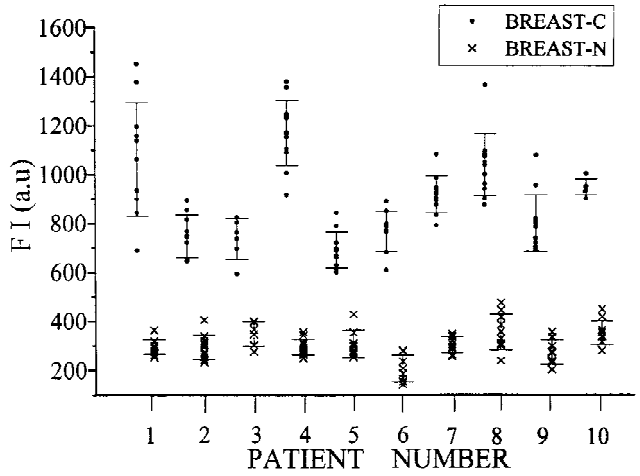

Fig. 4. Scatter plot for the integrated fluorescence intensity of paired cancerous and adjoining normal breast tissue samples from the first 10 patients included in the histogram shown in Figure 3.

ous sites from benign and normal tissue sites. In our samples, this ratio was observed to be considerably different for the two types of the cancerous and the benign breast tissue samples. The ratio was ~3.5 ± 1.2 for the Type-A cancerous tissues and 9.8 ± 1.5 for the Type-B cancerous tissues. Similarly, the ratio was found to be ~5.1 ± 1.7 for Type-A benign tumors and ~15.2 ± 3.5 for Type-B benign tumors. For the normal tissues, the ratio was observed to be ~2.9 ± 2. The ratio (I[340]/I[440]) discriminated the Type-B cancerous tissues from normal tissues, and Type-B benign tumors with the sensitivity and specificity approaching 100%. The discrimination was, however, poor when the total sample size was considered. Interestingly, for both types of cancerous tissues, the spectrally integrated intensity was

TABLE 1. Mean Value ± Standard Deviation of the Statistically Significant Discrimination Parameters for Breast Tissues

Discrimination parameter	Cancer	Benign	Normal
∑I	923.1 ± 219.8	286.2 ± 86.4	327.6 ± 80.3
I (BG)/I (VB)	1.240 ± 0.333	0.698 ± 0.239	0.806 ± 0.286

observed to be significantly more compared to the surrounding normal tissues with the ratio exceeding 4. However, no significant differences were observed in the spectrally integrated intensity of benign tumors and cancerous tissues. Therefore, the spectrally integrated fluorescence intensity could discriminate between cancerous and normal, and not between cancerous and benign.

In contrast, for 337 nm excitation, the spectrally integrated fluorescence intensity of cancerous tissue was larger than the spectrally integrated fluorescence intensities of normal, as well as benign tissues, and could provide a good discrimination. In order to analyze the diagnostic content of the breast tissue fluorescence spectra, a stepwise MVLR analysis of the 337 nm excited spectra was carried out. The input parameters used included the spectrally integrated fluorescence intensity (∑I), ratio of normalized intensities at wavelength pairs of equal blood absorption (I[440]/I[380], I[450]/I[550], I[480]/I[525], in order to take care of the likely interference from blood absorption [11]), values for normalized fluorescence intensities at 390 nm (I[390]) and 480 nm (I[480]), and ratio of integrated fluorescence intensities over the wavelength bands 450nm–550nm (BG) and 360nm–420nm (VB), I[BG]/I[VB].

For discriminating cancerous from normal tissues, only ∑I was found to contribute significantly, but for discriminating cancerous from benign tumors, two parameters viz. ∑I and I[BG]/I[VB] were found to contribute. However, very good discrimination was possible in the latter case also by using a discrimination score based on ∑I only. This is interesting because having a discrimination parameter based only on ∑I will considerably simplify the experimental arrangement, since no spectral resolution is required. The mean values ± standard deviation of the ∑I values, as well as of I[BG]/I[VB] values are listed in Table 1. In Figures 5 and 6, we show the scatter plot of the discrimination scores for cancerous, normal, and benign breast tissues based on ∑I alone. Sensitivity and specificity values of ~98% were achieved

TABLE 2. Sensitivity, Specificity, Predictive Value Positive, and Predictive Value Negative for Discriminating Cancerous From Adjoining Normal and Benign Breast Tissues Using the Spectrally Integrated Intensity Values and the 2-Variable MVLR Scores*

Discrimination goal	Discrimination parameter	Sensitivity (%)	Specificity (%)	Predictive value positive (%)	Predictive value negative (%)
C vs. N	∑I	98.8	98.7	98.8	98.7
	MVLR score	98.8	98.7	98.8	98.7
C vs. B	∑I	99.6	97.9	96.4	99.8
	MVLR score	99.6	98.4	97.2	99.8

*The cut-off point used was the weighted average of the $\bar{x} + \sigma$ and $\bar{x} - \sigma$ of the respective discrimination scores for the tissue types.

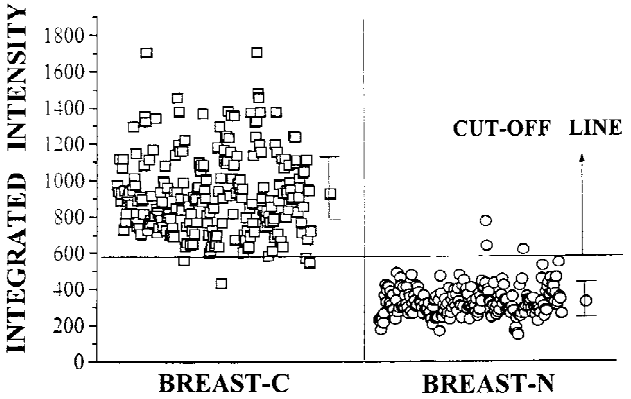

Fig. 5. Scatter plot for the spectrally integrated intensity (∑I) of cancerous (BREAST-C) and adjoining normal (BREAST-N) breast tissues.

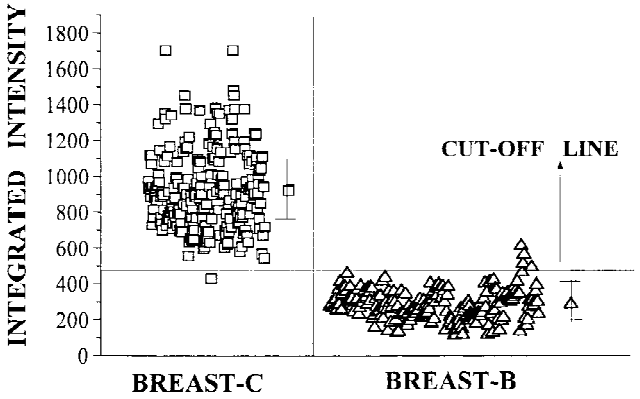

Fig. 6. Scatter plot for the spectrally integrated intensity (∑I) of cancerous (BREAST-C) and benign (BREAST-B) breast tissues.

for discriminating cancerous from adjoining normal breast tissues, as well as benign tumors over the sample size investigated. Table 2 lists the sensitivity, specificity, predictive value positive, and predictive value negative of the ∑I values using the weighted average of $\bar{x} + \sigma$ and $\bar{x} - \sigma$ of the respective discrimination scores for the tissue types. The corresponding values achieved with 2-variable MVLR analyses are also shown.

CONCLUSION

To conclude, our in vitro studies on human breast tissues show that a discrimination parameter based on absolute intensity alone can provide a very good discrimination between cancerous, normal, and benign breast tissues. The results, therefore, suggest that a straightforward measurement of the total integrated fluorescence intensity as opposed to the analysis of the fluorescence spectra can provide excellent discrimination.

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